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for Cytotoxic Anti-Angiogenesis Therapy

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We hypothesized that tumor-associated endothelium can be targeted with chemotherapy-loaded immunoliposomes (ILs) directed against VEGF Receptor 2 (VEGFR2, Flk-1, KDR). VEGFR2 is the primary receptor for VEGF-mediated tumor angiogenesis, and as a cell surface receptor on endothelial cells provides a highly accessible target for immunoliposome therapy. Accordingly, we have developed anti-VEGFR2 ILs to deliver highly potent chemotherapeutics to VEGFR2-expressing endothelial cells for cytotoxic antiangiogenic treatment. Cloned anti-VEGFR2 scFv 4G7 sequences were expressed in E. coli, and purified scFv containing a C-terminal cysteine residue were conjugated to liposomes using three different strategies. The resulting stable constructs bound specifically to recombinant VEGFR2 in ELISA and BIAcor assays, and to VEGFR2-expressing endothelial cell lines (HUVEC, BEND3) and 293/VEGFR2 transfectants in flow cytometric and immunofluorescence assays. Anti-VEGFR2 ILs binding was followed by endocytosis, resulting in intracellular drug delivery. In contrast, non-targeted control liposomes failed to bind or internalize. Anti-VEGFR2 ILs were loaded with various chemotherapeutics for targeted delivery to VEGFR2-expressing cells in vitro. Anti-VEGFR2 ILs containing doxorubicin (dox) showed potent cytotoxicity against BEND3 cells but not non-VEGFR2-expressing SK-BR-3 cells, indicating selective and efficient delivery. ILs containing alternate compounds such as vinorelbine, aminoellipticine, swainsonine, and breflate have also been generated.

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INTRODUCTION

Immunoliposomes (ILs) represent a strategy for tumor-targeted drug delivery by conjugation of monoclonal antibody fragments to liposomes (1). In studies that were partially supported by a previous DOD Career Development Award (DAMD17-94-J-4195), we developed anti-HER2 ILs in which Fab' (2, 3) or scFv fragments (4) were covalently linked to long circulating, sterically stabilized liposomes. Anti-HER2 immunoliposomes bound efficiently to and internalized in HER2-overexpressing cells *in vitro* and *in vivo*, resulting in intracellular drug delivery (2, 3, 5). In HER2-overexpressing tumor xenograft models, anti-HER2 immunoliposomes loaded with doxorubicin displayed potent and selective anticancer activity, including significantly superior efficacy vs. all other treatment conditions tested: free doxorubicin, liposomal doxorubicin, free MAb (trastuzumab/Herceptin®), and combinations of free doxorubicin or liposomal doxorubicin plus free MAb (4). As a result of these studies, anti-HER2 immunoliposomes are currently undergoing scale up for Phase I clinical studies.

In this project, we hypothesized that tumor-associated endothelium can be targeted with immunoliposomes (ILs) directed against VEGF Receptor 2 (VEGFR2, Flk-1, KDR). VEGFR2 is the primary receptor for VEGF-mediated effects during tumor angiogenesis, and as a cell surface receptor on endothelial cells provides a highly accessible target for immunoliposome therapy. Immunoliposome-mediated drug delivery to tumor vasculature, unlike most antiangiogenic therapies, is cytotoxic rather than cytostatic. Accordingly, this project seeks to develop anti-VEGFR2 ILs to deliver chemotherapeutic agents to VEGFR2-expressing endothelial cells involved in tumor angiogenesis, to achieve cytotoxic antiangiogenic treatment.

BODY [contains unpublished data]

Task 1. Preparation of anti-Flk-1 ILs for angiogenesis targeting.

A. Preparation of anti-VEGFR2 (Flk-1) immunoliposomes.

1. Selection of monoclonal antibodies (MAbs).

As described in our Year 1 Report, we have generated anti-VEGFR2 ILs containing scFv 4G7. 4G7 shows cross-species specificity for both human (KDR) and mouse (flk-1) receptor. Hence, 4G7-containing ILs are amenable for use in preclinical studies in nude mouse models, in which flk-1-expressing murine endothelial cells support angiogenesis of human tumor xenografts. If warranted, the same construct can proceed to clinical studies in humans against KDR-expressing tumor endothelium. Another advantage is that recombinant scFv, as opposed to hybridoma-derived MAbs, can be rapidly re-engineered for site-specific and efficient immunoliposome conjugation.

2. Preparation of MAb fragments.

For immunoliposome conjugation, the gene encoding scFv 4G7 was re-engineered to provide specific covalent linkage to modified liposomes. scFv 4G7 sequences were subcloned into a bacterial expression vector (pELK) with additional sequences added downstream of the multiple cloning site. These sequences included: a c-myc epitope tag, a single cysteine residue for thiol linkage to liposomes, and (his)$_6$ for affinity purification. The resulting scFv 4G7-cys construct was confirmed by DNA sequencing, and then expressed in E. coli and purified using nickel-based affinity chomatography. Yields were initially low (<1 mg/L), but were subsequently optimized using benchtop procedures to 2-5 mg/L. Purified scFv was then subjected to cysteamine reduction for subsequent conjugation.

3. Construction of anti-VEGFR2 immunoliposomes.

Anti-VEGFR2 scFv was conjugated to liposomes using three different strategies (Fig. 1). Each of these provided highly efficient linking of scFv to lipid-based anchors; single site attachment on the scFv, assuring preservation of the scFv binding site and proper scFv orientation; and multiple attachment sites on liposomes, resulting in highly avid multivalent binding particles (~30 scFv per liposome).

A) MAb-PEG conjugation.

Liposomes were prepared as small unilamellar vesicles (70-100 nm) containing hydrogenated soy phosphatidylcholine (HSPC) and cholesterol (Chol) as described (2). The high transition temperature of HSPC (T_m = 54 °C) induced the liposome bilayer into the solid or gel phase at 37°C. Polyethylene glycol (M_r = 1900)-derivatized distearoyl-phosphatidylethanolamine (PEG-PE) was present at 7 mol% of total lipid for steric stabilization. The PEG-PE included 1 mol% of maleimide-modified PEG-PE (M-PEG-PE), in which the maleimide group at the distal terminus of the PEG chain provides a specific attachment site for the MAb fragment. These sterically stabilized liposomes provide very long circulation times and are non-immunogenic (1).

scFv 4G7-cys was chemically reduced, and then covalently conjugated to sterically stabilized liposomes via disulfide linkage to M-PEG-PE included within the liposome (Fig. 1A). In our previous studies, anti-HER2 MAb fragments rhuMAbHER2-Fab', scFv C6.5, and scFv F5 could be conjugated to liposomes with high efficiency (>80% of MAb fragment added). However, conjugation of 4G7-cys displayed variable efficiency, in part due to incomplete reduction. In addition to the added cys residue at the C-terminius, there are 4 additional cys residues within the scFv 4G7-cys sequence. Controlled reduction was necessary to ensure ~1 reduced thiol group per scFv molecule, corresponding to the reduced C-terminal cysteine residue. Lower ratios indicated a suboptimal yield of available scFv for conjugation, while higher ratios were associated with the undesirable reduction of internal disulfides. Because of this variability, conjugation of scFv 4G7 required further optimization, including evaluation of alternative conjugation procedures.

B) Micellar incorporation.

We have developed a newer conjugation strategy involving two-step conjugation of scFv to a soluble linker (M-PEG-PE), followed by incorporation into preformed liposomes. In the first step, reduced scFv 4G7-cys was again covalently linked via thiol chemistry to M-PEG-PE, but the reaction here proceeds in solution to form mixed micelles. The resulting micellar immunoconjugate is then incorporated into liposomes under conditions of controlled heating (Fig. 1B). The resulting ILs have the same final structure as in (A). This versatile strategy allows existing liposomes of various compositions to be converted into immunoliposomes, thus obviating the need to prepare dedicated liposomes containing specific linkage sites for MAb fragments. For example, we have used this strategy to conjugate anti-HER2 scFv to commercial preparations of PEGylated liposomal doxorubicin (Doxil®), thus transforming an existing liposomal drug into an immunotargeted version with specificity against HER2-overexpressing cancer cells (6).

We have applied this procedure to scFv 4G7-cys. Conjugation to M-PEG-PE in solution occurred with high efficiency. The resulting 4G7-M-PEG-PE conjugates were then incorporated into liposomes, including various liposomal drugs prepared in our laboratory as well as commercial Doxil, also with high efficiency (Fig. 2).

C) Nickel chelation.

We have developed a procedure for rapid coupling of scFv containing (his)$_6$ to liposomes in which Ni++ is stably chelated at the liposome surface (Fig. 1C; Nielsen et al., submitted). It is unlikely that these chelated ILs will provide sufficient stability under in vivo conditions as compared with ILs containing MAb fragments covalently linked to M-PEG-PE, which we have shown to be capable of long circulation as intact constructs in rats (Park et al., submitted). However, the chelation method circumvents the requirement for thiol reduction and provides extremely rapid, single-step conjugation that is ideal for in vitro screening assays.

We have generated anti-VEGFR2 ILs by nickel chelation of scFv 4G7 to liposomes containing fluorescent markers or various anticancer compounds (see below).

Our previous studies have included further re-engineering of two different anti-HER2 scFv's from pELK into an alternative expression vector suitable for scaled up GMP manufacturing. This clinically compatible expression system includes a bacterial alkaline phosphatase promoter rather than *lacZ*, and a kanamycin rather than ampicillin drug resistance marker. In collaboration with the NCI Biological Resources Branch (NCI BRB), current yields of scFv F5 have reached 150 mg/L fermentate at the 80L scale, which is amply sufficient for human clinical trials. Methods for scFv purification and immunoliposome conjugation using micellar incorporation have also been scaled up for GMP production. Anti-HER2 ILs are anticipated to enter Phase I clinical trials in 2001. Hence, scaled up expression/purification of anti-VEGFR2 ILs is likely to be quite feasible, as protocols have already been established for the similar anti-HER2 ILs construct.

B. Binding studies.

Anti-VEGFR2 ILs were evaluated for binding to soluble VEGFR2 by surface plasmon resonance in a BIAcore1000 instrument (BIAcore Inc.). Recombinant VEGFR2 fusion protein was coupled to a CM5 sensor chip, and binding rates measured under continuous flow of 15 µL/min. ILs were injected at a concentration of 50 µM phospholipid. Binding activity was determined from a linear standard curve of binding slope versus concentration. This demonstrated specific binding of anti-VEGFR2 ILs to purified VEGFR2, whereas there was no detectable binding by liposomes lacking scFv. Binding of multivalent ILs to VEGFR2 was highly avid, with $k_{off} = 6.7 \times 10^{-4}$/sec. This result predicts a binding half-life ($t_{1/2}$) for anti-VEGFR2 ILs to purified antigen of 17 min, assuming simple exponential decay (e^{-kt}, where $k = k_{off}$).

C. Internalization and intracellular disposition studies in vitro.

Fluorescence microscopy was performed to evaluate binding and internalization of anti-VEGFR2 ILs in VEGFR-expressing cells. Initial studies used anti-VEGFR2 ILs that were loaded with pyranine (1-hydroxypyrene-3,6,8-trisulfonic acid, HPTS), a pH-sensitive fluorophore that can be readily and stably encapsulated in liposomes (3). Incubation of flk-1-expressing BEND 3 endothelial cells with HPTS-loaded ILs for 4 or 12 h demonstrated cell surface binding and extensive accumulation of ILs intracellularly, predominantly in the cytoplasm and consistent with internalization via endocytosis. In contrast, incubation with control liposomes (prepared identically except for omission of scFv conjugation) or irrelevant anti-HER2 ILs showed no detectable binding or intracellular uptake.

Additional studies performed in Year 2 have used rhodamine-labeled anti-VEGFR2 ILs to further evaluate immunoliposome uptake. In addition to flk-1-expressing BEND3 endothelial cells, 293/KDR cells (293 cells stably transfected with KDR) and, as a negative control, non-VEGFR2-expressing SK-BR-3 breast cancer cells were also included. Treatment of BEND3 or 293/KDR cells with rhodamine-labeled anti-VEGFR2 ILs for 2, 4, 8, or 12 h resulted in marked accumulation of ILs within the cytoplasm (Fig. 3). Control liposomes (rhodamine-labeled sterically stabilized liposomes, identical to ILs except for omission of scFv) and irrelevant anti-HER2 ILs showed no detectable uptake in BEND3 or 293/KDR cells. Treatment of 293 cells not transfected with KDR or SK-BR-3 cells with anti-VEGFR2 ILs failed to show any detectable uptake.

Task 2. Evaluation of cytotoxicity in vitro.

A. Preparation of drug-loaded ILs: doxorubicin, vinorelbine, ellipticine, swainsonine, breflate.

Doxorubicin (dox) was encapsulated into liposomes with high efficiency by remote loading. In this strategy, formation of a pH gradient across the liposome membrane induces protonation and ion trapping of dox within the aqueous interior of liposomes, ultimately producing gelation or crystallization of 10^4 dox molecules per liposome (7). Anti-VEGFR2 ILs were loaded with dox using this method, with efficiencies of encapsulation of 90-100% of added dox. As an alternative to dox-loading of ILs in our laboratory, we also obtained commercial PEGylated liposomal doxorubicin (Doxil), and converted these to ILs using the micellar incorporation method described above.

In addition to doxorubicin, we prepared ILs containing other cytotoxic agents, including other approved chemotherapeutics (vinorelbine) as well as novel anticancer drugs. These various immunoliposome constructs will allow us to study the effects of multiple drugs, with diverse mechanisms of action, when targeted to endothelial cells.

Sterically stabilized liposomes containing encapsulated vinorelbine (VRL; Navelbine®) were developed using a modified remote loading technique (8). VRL-loaded liposomes were stable after prolonged storage and showed minimal drug release in serum. Liposomal vinorelbine was used to generate anti-HER2 ILs-VRL, which showed significant antitumor activity *in vitro* and *in vivo*. Similarly, liposomal VRL was converted into anti-VEGFR2 ILs-VRL by micellar incorporation of scFv 4G7-M-PEG-PE.

Although we initially planned to prepare ILs containing additional cytotoxic drugs such as platinum-rhodamine and edatrexate, we modified this subaim to include newer and more promising drugs for liposome/immunoliposome encapsulation. In collaboration with the National Cancer Institute Developmental Therapeutics Program (NCI DTP), we have identified agents with unique mechanisms of action and highly potent activity against breast and other cancers in model systems; but which have not yet achieved clinical use because of pharmacologic limitations such as solubility/bioavailability, biodistribution, pharmacokinetics, and host toxicity. Several such compounds with chemical structures suitable for efficient encapsulation in liposomes/immunoliposomes were obtained from the NCI DTP (Fig. 4). These "problem" compounds are therefore ideal candidates for delivery via ILs, which can in principle provide efficient formulation, extended circulation time, direct delivery to target cells with internalization and intracellular release, and, consequently, enhanced therapeutic index.

The ellipticines, alkaloids derived from the Apocynaceae family, intercalate in DNA and induce topoisomerase II-mediated DNA damage via a mechanism distinct from anthracyclines or other existing topoisomerase inhibitors (9). Ellipticines have demonstrated very potent activity in preclinical screens,

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but also severe toxic effects in animal and human studies. In clinical trials, the semisynthetic derivative elliptinium was associated with hemolysis and allergic reactions, which were correlated with aggregation in the formulation. Nephrotoxicity was also prominently observed, and was associated with direct tubular injury. These factors greatly restrict the dosing of ellipticines, and led to the discontinuation of ellipticine development in the US, although low dose elliptinium was approved in Europe for the treatment of advanced breast cancer. The pharmacologic limitations of the ellipticines provide a strong rationale for immunoliposome encapsulation, which can in principle minimize both aggregation-related toxicities as well as renal tubular exposure.

For ellipticine encapsulation into liposomes, aminoellipticine was selected as an optimal derivative, and a procedure for remote loading developed. While aminoellipticine retains the potent anticancer activity of the ellipticines, the addition of the amino group allows for fast protonation and ion trapping within liposomes. Aminoellipticine was obtained from the NCI DTP, and encapsulated into liposomes using a remote loading approach. Encapsulation efficiency was 90-100%. Liposomal aminoellipticine was then converted to anti-VEGFR2 ILs by nickel chelation of 4G7.

Swainsonine (NSC 652469) is an alkaloidal mannosidase inhibitor that is highly active in multiple preclinical models of cancer (10). Clinical studies of swainsonine indicated rapid clearance and multiple systemic toxicities. Immunoliposome delivery provides a strategy for extending circulation and directly targeting swainsonine to antigen-expressing cells. Swainsonine, obtained from the NCI DTP, was encapsulated in liposomes and ILs using the remote loading technique, with efficiency of encapsulation of 90-100%.

The brefeldins are extremely potent cytotoxins with a novel mechanism of action. Brefeldin A (NSC 89671) is a commonly used laboratory reagent for its profound effects on protein processing and transport. In animals, brefeldin A is poorly soluble and rapidly cleared. Breflate (NSC 656202), an esterified prodrug of brefeldin A, is more water-soluble; however, in animal studies it is also rapidly cleared, and displays significant neurotoxicity and local tissue injury at the injection site. Breflate was encapsulated in liposomes and ILs using a remote loading technique, with efficiency of encapsulation of 83-95%.

B. Cytotoxicity in monolayer culture.

The cytotoxicity of various anti-VEGFR2 ILs prepared in Task 3A was evaluated in VEGFR2-expressing BEND3 cells in vitro.

1. Anti-VEGFR2 ILs-dox.

We previously showed that dox-loaded anti-HER2 ILs (anti-HER2 ILs-dox) were capable of highly efficient in vitro drug delivery. Remote loading allows encapsulation of 10,000 – 15,000 dox molecules per immunoliposome particle. Anti-HER2 ILs can accumulate in vitro to 23,000 liposomes per target cell (3), and thus the amount of dox that can be delivered via ILs is potentially 10^8 dox per cell. Given this enormously productive delivery, we hypothesized that immunoliposome delivery of dox to HER2-overexpressing target cells may match the efficiency with which free dox, a small (M_r 544) amphipathic molecule, readily diffuses through the cell membrane in vitro. In contrast, in cells that do not overexpress HER2, ILs delivery would be much less cytotoxic than free dox, and a large selective differential would be achieved. As predicted, treatment of SK-BR-3 cells for 1 hour with anti-HER2 ILs-dox yielded dose-dependent cytotoxicity comparable to that of free dox, indicating that immunoliposome delivery of dox was as efficient as the rapid diffusion of free dox into cells in vitro(2). Anti-HER2 IL-dox was 30-fold more cytotoxic than dox-loaded control ILs bearing irrelevant MAb, which affected cell growth only at very high concentrations due to leakage of free dox.

Anti-VEGFR2 ILs-dox were prepared by micellar incorporation of 4G7-M-PEG-PE into Doxil, and incubated with BEND3 endothelial cells for 4 h at 37 °C (Fig. 5A). Dose-dependent cytotoxicity was observed, with immunoliposome-mediated delivery approximately equivalent to the rapid diffusion of free dox into the cells (IC50 = 0.5 µg/ml for both ILs-dox and free dox). In contrast, anti-VEGFR2 ILs-dox was approximately 100-fold more potent than control liposomal dox (Doxil), from which ILs had been prepared. In non-VEGFR2-expressing SK-BR-3 cells, ILs were no more potent than control liposomal dox, with both ILs and liposomes showing markedly less activity than free dox (Fig. 5B).

Taken together, these studies indicated that anti-VEGFR2 ILs were capable of highly efficient delivery of dox to target cells, and that this delivery was selective for VEGFR2 expressing cells.

2. Anti-VEGFR2 ILs-VRL.

Anti-VEGFR2 ILs containing vinorelbine (VRL) were prepared by the nickel chelation method, and incubated with BEND3 cells for 4 h at 37 °C (Fig. 5C). ILs-VRL was approximately equivalent to free VRL, and possibly showed increased potency at higher concentrations. Delivery via ILs was at least 30-fold more efficient than control liposomal VRL, prepared identically except for omission of scFv.

3. Anti-VEGFR2 ILs-AE.

Anti-VEGFR2 ILs containing aminoellipticine (AE) were prepared by the nickel chelation method, and incubated with BEND3 cells for 4 h at 37 °C (Fig. 5D). Anti-VEGFR2 ILs-AE showed at least equivalent cytotoxicity as free AE. However, delivery via ILs was only approximately 3-fold more efficient than via control liposomal AE. The relatively high cytotoxicity of liposomal AE was likely due to leakage of free AE from the liposomes, thus confounding the comparison of immunoliposome vs. liposome delivery, and also indicating problems with the stability of liposomal AE. We are therefore attempting to optimize the liposome formulation of AE, including alternative conjugation strategies (LSs-MAb linkage or micellar incorporation rather than nickel chelation) and alternative phospholipid components.

4. Other Anti-VEGFR2 ILs

Cytotoxicity of anti-VEGFR2 ILs containing other compounds, including swainsonine and breflate, are in progress.

Task 3. Biodistribution, tumor localization, and PK studies in animal models.

A. PK studies in normal rats.

The plasma pharmacokinetics (PK) of immunoliposomes constructed using our MAb-PEG conjugation strategy was performed in normal Sprague-Dawley rats. Dox-loaded ILs were prepared with 0-7 mol% PEG and with MAb fragments (anti-HER2) linked to the termini of PEG chains (MAb-PEG linkage). After a single i.v. dose, all ILs-dox constructs showed a biphasic plasma PK profile for dox (Fig. 1A), with terminal $t_{1/2}$ = 11.6 - 13.6 h, AUC = 57,539 - 93,133 min%, and mean residence time (MRT) = 15.8 - 24.3 h. In contrast, dox levels were undetectable beyond 5 min following administration of the equivalent dose of free dox, demonstrating a marked pharmacokinetic advantage for immunoliposome-encapsulated dox (Fig. 1A). Direct comparison of ILs-dox vs. sterically stabilized liposomal dox prepared identically except for omission of MAb fragments showed indistinguishable PK. The long circulation times of ILs-dox are also equivalent to previous reports of sterically stabilized liposomal dox, and greatly superior to those of "conventional" liposomes (11). Hence, the presence of MAb fragments on ILs-dox did not measurably alter clearance in normal rats, in contrast to results obtained with many other immunoconjugates in vivo (1).

Another major limitation of many immunoconjugate strategies has been the immunogenicity of the construct, as either the MAb component, effector/cytotoxic component, or linker can elicit a host immune response that precludes repeated administrations. In order to circumvent this problem, our ILs were designed to have components of minimal immunogenicity: small scFv fragments linked to sterically stabilized liposomes. MAb fragments rather than intact IgG were used to eliminate the potential for immune recognition associated with Fc sequences, and . To confirm that repeated administrations of these ILs do not lead to accelerated clearance, multiple dose PK studies were performed in normal rats. Radiolabeled anti-HER2 immunoliposomes were administered once a week for three doses, using the same lipid dose and schedule as in therapy studies (Park et al., submitted). Anti-HER2 ILs showed equivalent plasma PK in rats following the third and final dose as in naive rats without prior immunoliposome treatment (Fig. 1B). Furthermore, anti-HER2 ILs after one or three doses produced plasma PK that was indistinguishable from that of sterically stabilized liposomes (prepared

identically except for omission of Fab' fragments). Thus, the presence of MAb fragments on ILs did not result in accelerated clearance over a multiple dose schedule. These results suggested that the construct design for these ILs succeeded in minimizing immunogenicity, at least over the duration of a therapeutic course.

We anticipate that the PK of anti-VEGFR2 ILs-dox will be equivalent to that of anti-HER2 ILs-dox, since the constructs are essentially identical except for scFv specificity. PK studies of anti-VEGFR2-dox are in preparation.

The PK of sterically stabilized liposomes containing aminoellipticine were evaluated following a single i.v. dose in normal rats. Liposomal AE showed prolonged circulation that markedly exceeded free drug. However, $t_{1/2}$ and mean residence time were significantly shorter than that of sterically stabilized liposomal dox, consistent with some degree leakage of AE from these long circulating liposomes during circulation. Optimization of stability is in progress.

B. Biodistribution/tumor localization studies.
Further in vivo studies are in preparation.

KEY RESEARCH ACCOMPLISHMENTS

- Anti-VEGFR2 scFv 4G7-cys has been engineered for efficient bacterial expression and immunoliposome conjugation. A similar procedure has been scaled up for GMP manufacturing, and thus the feasibility of further development of this construct has been demonstrated.
- Purified recombinant scFv 4G7-cys binds to both murine (Flk-1) and human (KDR) homologues of VEGFR2 in ELISA, surface plasmon resonance (BIAcor), and flow cytometry assays.
- scFv 4G7-cys has been conjugated to sterically stabilized liposomes to generate anti-VEGFR2 immunoliposomes (ILs).
- Anti-VEGFR2 ILs bind avidly to purified recombinant VEGFR2 in surface plasmon resonance (BIAcor) assay and to VEGFR2-expressing cells in flow cytometry assay.
- Binding of anti-VEGFR2 ILs to VEGFR2-expressing BEND3 endothelial cells results in internalization and intracellular accumulation.
- Anti-VEGFR2 ILs containing doxorubicin have been generated. These show highly efficient cytotoxicity against BEND3 cells.
- Anti-VEGFR2 ILs containing vinorelbine have been generated. These show highly efficient cytotoxicity against BEND3 cells.
- Anti-VEGFR2 ILs containing aminoellipticine have been generated. These appear to show efficient cytotoxicity against BEND3 cells, although further optimization of liposome encapsulation is required.
- ILs containing swainsonine have been generated.
- ILs containing breflate have been generated.
- ILs produced by these methods are long-circulating in vivo.

REPORTABLE OUTCOMES

Greiser U, Kirpotin D, Hong K, Nielsen U, Marks JD, Zhu Z, and Park JW. Development of anti-VEGFR2 immunoliposomes. *Era of Hope DOD Breast Cancer Res. Program Meeting*, Atlanta, GA, June 8-11, 2000.

CONCLUSIONS

We hypothesized that tumor-associated endothelium can be targeted with chemotherapy-loaded immunoliposomes (ILs) directed against VEGF Receptor 2 (VEGFR2, Flk-1, KDR). VEGFR2 is the primary receptor for VEGF-mediated effects during tumor angiogenesis, and as a cell surface receptor on endothelial cells provides a highly accessible target for immunoliposome therapy. Immunoliposome-mediated drug delivery to tumor vasculature, unlike most antiangiogenic therapies, is cytotoxic rather than cytostatic.

Accordingly, we have generated anti-VEGFR2 ILs to deliver chemotherapeutic agents to VEGFR2-expressing endothelial cells involved in tumor angiogenesis, to achieve cytotoxic antiangiogenic treatment. Cloned anti-VEGFR2 scFv 4G7 sequences were expressed in E. coli, and purified scFv containing a C-terminal cysteine residue were covalently conjugated via disulfide linkage to modified phospholipids incorporated in sterically stabilized liposomes. Initial variability of conjugation presented an initial obstacle that was overcome by the development of two new conjugation strategies involving micellar incorporation or nickel chelation.

The resulting stable constructs bound specifically to recombinant VEGFR2 in surface plasmon resonance (BIAcor) assay. In binding and internalization studies using ILs containing fluorescent probes, anti-VEGFR2 ILs bound specifically to VEGFR2-expressing endothelial cell lines, including Flk-1-expressing murine endothelial cells (BEND3) and KDR-expressing 293 stable transfectants. Anti-VEGFR2 ILs binding was followed by endocytosis, resulting in intracellular drug delivery. In contrast, non-MAb-containing sterically stabilized liposomes as well as irrelevant anti-HER2 ILs failed to show detectable binding and did not accumulate intracellularly. Anti-VEGFR2 ILs did not show detectable binding to SK-BR-3 breast cancer cells or non-transfected 293 cells, both of which lack VEGFR2 expression.

Anti-VEGFR2 ILs were loaded with a variety of cytotoxic drugs for targeted delivery to VEGFR2-expressing endothelial cells in vitro. Anti-VEGFR2 ILs containing doxorubicin (dox) were produced by micellar incorporation of scFv into commercial liposomal dox. The resulting anti-VEGFR2 ILs-dox showed potent cytotoxicity against VEGFR2-expressing BEND3 cells, but not to SK-BR-3 cells; thus, anti-VEGFR2 ILs were able to mediate selective and efficient drug delivery.

ILs were also prepared with encapsulated vinorelbine, aminoellipticine, swainsonine, or breflate. Anti-VEGFR2 ILs loaded with vinorelbine showed efficient delivery in BEND3 cells.

The significance of the work to date is that the feasibility of immunoliposomes specifically directed against VEGFR2 has been demonstrated. Furthermore, efficient methods to produce these immunoliposomes and to load them with a variety of anticancer compounds have been established. These constructs will be further studied in preclinical models to assess the efficacy of immunotargeted drug delivery to VEGFR2-expressing cells.

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APPENDICES

FIGURE LEGENDS [contains unpublished data]

Figure 1. Schematic representation of anti-VEGFR2 immunoliposomes (ILs).

A) MAb-PEG linkage. scFv 4G7-cys was covalently conjugated to liposomes containing M-PEG-PE. Thiol linkage between the C-terminal cysteine with maleimide moities on the termini of PEG chains provide specific single-site attachment on the scFv and multiple attachment sites on liposomes.

B) Micellar incorporation. Using the same linkage chemistry as in (A), a two-step procedure was employed to modify preformed liposomes lacking M-PEG-PE into ILs. scFv 4G7-cys was covalently conjugated to M-PEG-PE in solution, followed by incorporation of the micellar immunoconjugate into liposomes under controlled heating. The resulting ILs have the same structure as in (A), but can be generated using various liposomal drugs as starting material, such as Doxil.

C) Nickel chelation. A rapid conjugation strategy was developed involving chelation-based linkage of scFv's, including phage antibody-derived scFv, to liposome. scFv 4G7-cys, which contains a $(his)_6$ sequence, was added to liposomes containing a surface chelator in the presence of nickel.

Figure 2. Anti-VEGFR2 ILs-dox produced by micellar incorporation of scFv 4G7-M-PEG-PE conjugate.

scFv 4G7-cys was expressed in E. coli, reduced, and conjugated to M-PEG-PE in solution. The scFv-M-PEG-PE conjugate was then incorporated into commerical PEGylated liposomal doxorubicin (Doxil) to generate anti-VEGFR2 ILs-dox. 10% SDS-PAGE, coomassie stained.

Lanes: 1) Molecular weight markers; 2) 4G7-cys after bacterial expression and purification; 3) 4G7-cys after reduction; 4) 4G7-M-PEG-PE conjugate, formed by addition of 4G7-cys and M-PEG-PE in solution; 5) mixture of 4G7-cys with liposomal doxorubicin (Doxil); 6-8) immunoliposomes after gel filtration; 9) Molecular weight markers #2.

Figure 3. Binding and internalization of antiVEGFR2 ILs in VEGFR-expressing endothelial cells.

Anti-VEGF2 ILs were formed by micellar incorporation of scFv 4G7-M-PEG-PE into liposomes containing rhodamine-PE. ILs were then incubated with 293/KDR, 293 cells stably transfected with KDR, at 37°C for 8 h. The same representative field of cells is shown at high power magnification under either fluorescence microscopy (A) or phase contrast microscopy (B).

Figure 4. Chemical structures of novel anticancer compounds encapsulated in immunoliposomes.

Novel compounds with potent anticancer activity were obtained from the National Cancer Institute Developmental Therapeutics Program (NCI DTP). All of these compounds have shown promising activity in in vitro and in vivo screens, and, in some cases, in clinical trials. However, all of these are "problem" compounds with various pharmacologic limitations that have impeded further drug development (see text). To circumvent these limitations, we have successfully encapsulated breflate, aminoellipticine, swainsonine, and morpholinoadriamycin into immunoliposomes.

Figure 5. Cytotoxicity of drug-loaded anti-VEGFR2 ILs in vitro.

Anti-VEGFR2 ILs were formed by incorporation of scFv 4G7-cys-M-PEG-PE (Fig. 1B) or by chelation of scFv 4G7-cys (Fig. 1C) into liposomes loaded with the anticancer compounds indicated. The indicated cell lines (VEGFR2-expressing BEND3 endothelial cells or non-expressing SK-BR-3 breast cancer cells) were incubated with the ILs for 4 h at 37 °C, then washed and assayed for cytotoxicity by MTT staining. Positive control was free drug, and negative control was liposomal drug prepared identically as ILs except for omission of scFv. MTT staining was normalized against that of mock treated cells incubated with media alone, which was defined as 100% cell growth.

A) Anti-VEGFR2 ILs-Dox treatment of BEND3 endothelial cells.
B) Anti-VEGFR2 ILs-Dox treatment of SK-BR-3 cells.
C) Anti-VEGFR2 ILs-Vinorelbine treatment of BEND3 endothelial cells.
D) Anti-VEGFR ILs-Aminoellipticine treatment of BEND3 endothelial cells.
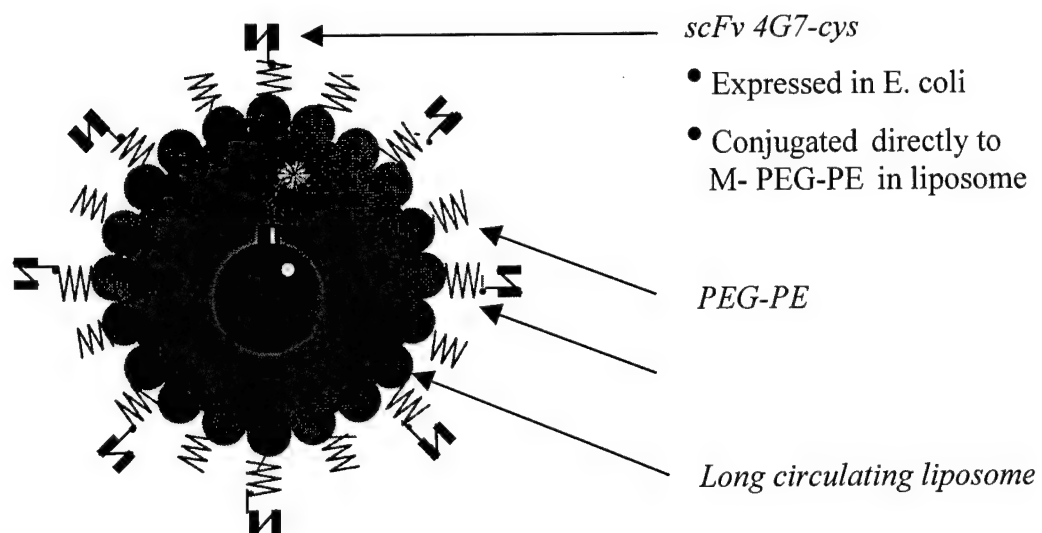
Figure 6. Plasma pharmacokinetics of ILs.

A) Plasma PK of ILs-dox vs. free dox following single i.v. injection in rats. ILs containing anti-HER2 MAb fragments and either 0 mol% PEG *(squares)* or 7 mol% PEG *(diamonds)* were compared to free dox *(circle)* following single i.v. dose at time 0. Dose of ILs was 5 μmol of total lipid, 0.9 mg of dox; free dox dose was 0.9 mg. Dox levels were determined at the indicated times and expressed as a percentage of the dox concentration at 5 min. Dox was undetectable in plasma beyond 5 min following injection of free dox.

B) Plasma PK of radiolabeled ILs following multiple vs. single i.v. injection in rats. ILs (6 mol% PEG, MAb-PEG linkage, anti-HER2 MAb fragments) were administered i.v. in rats every week for 3 weeks as in the therapy studies. For the third dose, ILs were loaded with ^{67}Ga-DTPA chelates, and immunoliposome levels determined at the indicated times by radioactivity counting *(triangles)*. For comparison, ^{67}Ga-DTPA-loaded ILs *(squares)* and sterically stabilized liposomes *(circles)* were also administered to naive rats. Plasma levels of ILs or liposomes are expressed as a percentage of the radioactivity present at 1 min.

FIGURE 1

A) MAb-PEG linkage

scFv 4G7-cys

- Expressed in E. coli
- Conjugated directly to M- PEG-PE in liposome

PEG-PE

Long circulating liposome

FIGURE 1

B) Micellar Incorporation

scFv 4G7-cys
- Expressed in E. coli
- Preconjugated to M-PEG-PE

scFv-M-PEG-PE
- Incorporated into liposome

Long circulating liposome
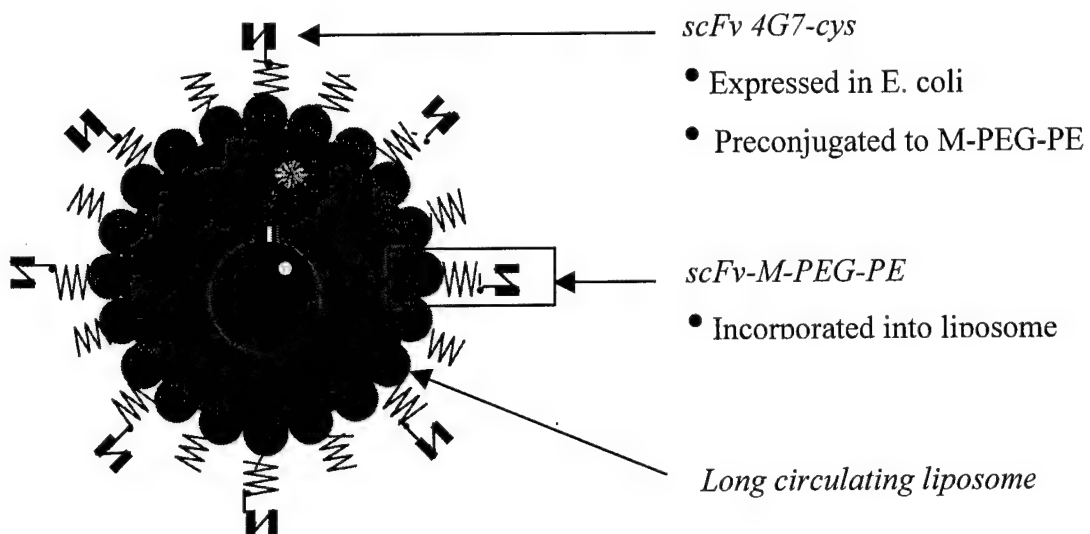
FIGURE 1

C) Nickel Chelation

FIGURE 2

4G7 Dimer
4G7 M-PEG-PE
4G7-cys
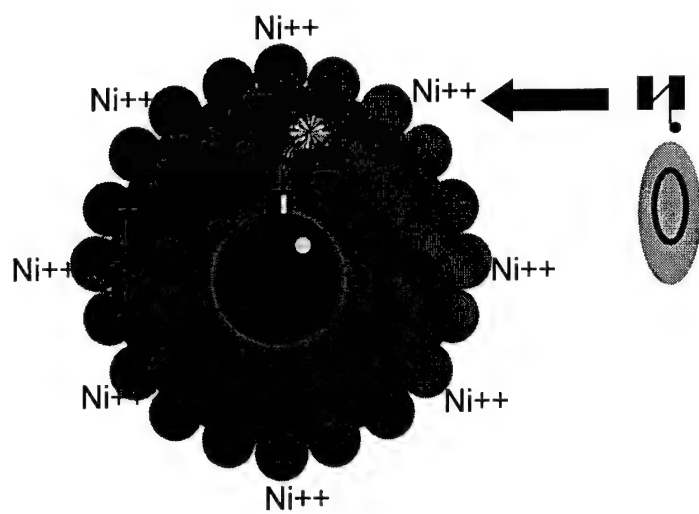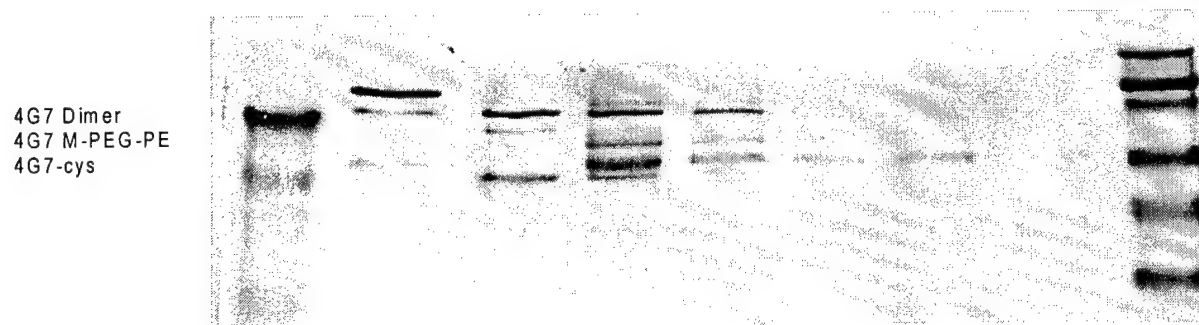
FIGURE 3

A) Fluorescence Microscopy
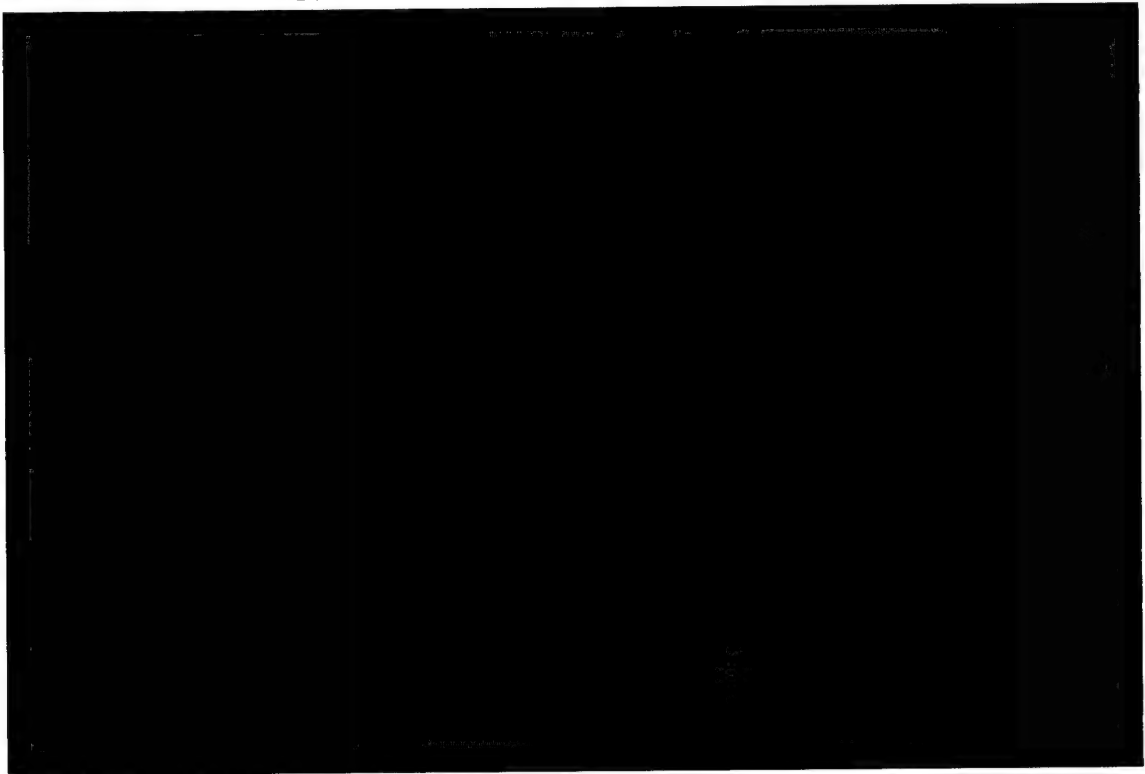
B) Phase Contrast Microscopy
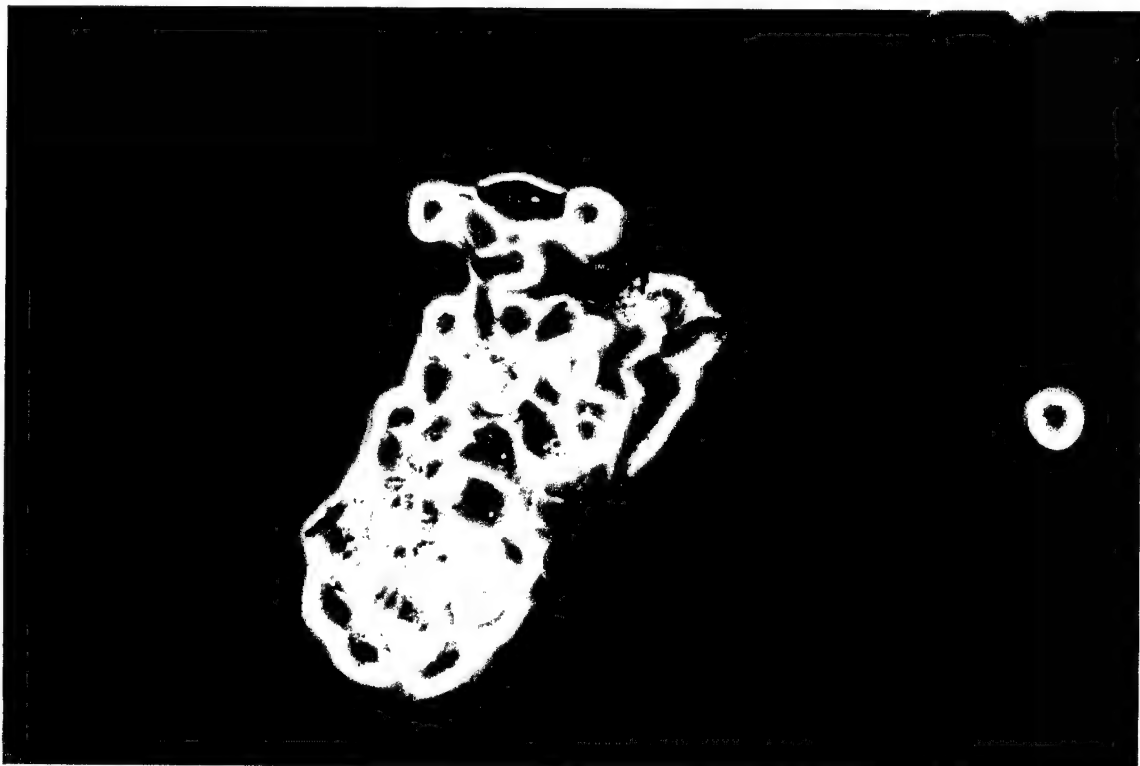
FIGURE 4
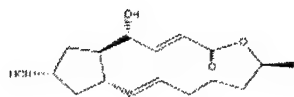
brefeldin A
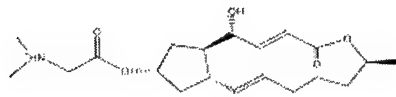
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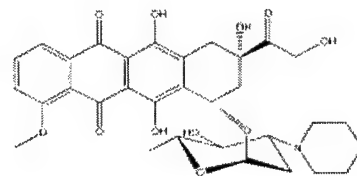
Morpholinoadriamycin
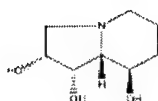
Swainsonine
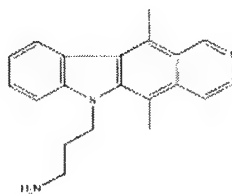
6-3-aminopropyl-ellipticine
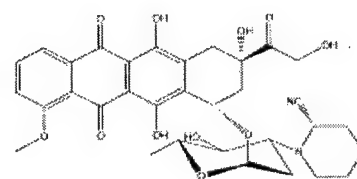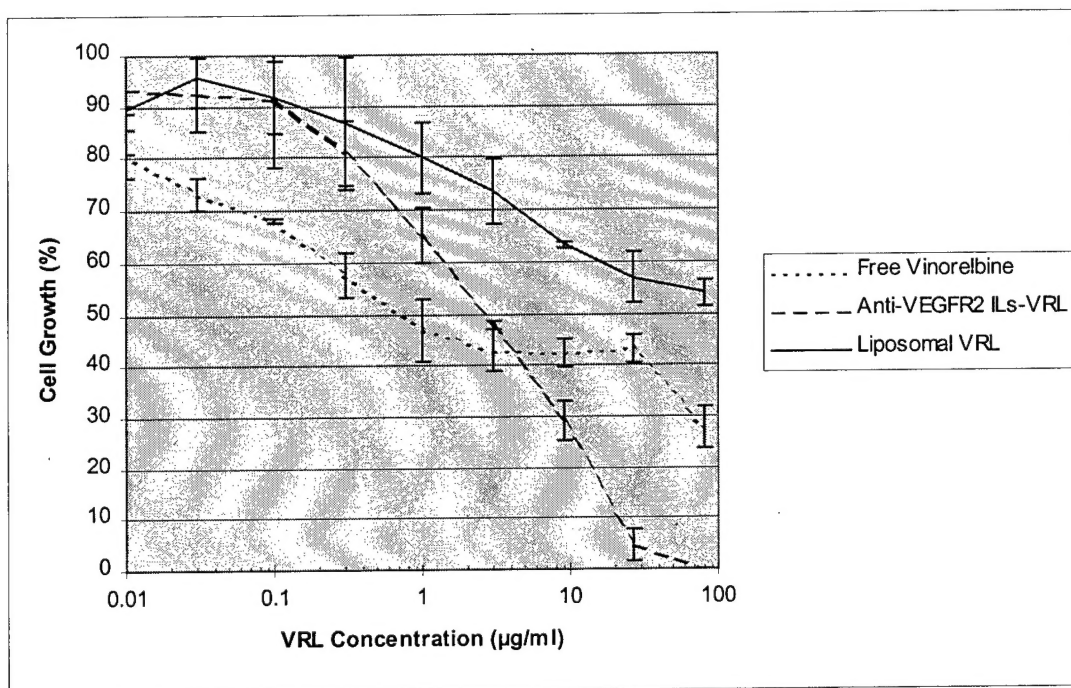

Cyanomorpholinoadriamycin

FIGURE 5A
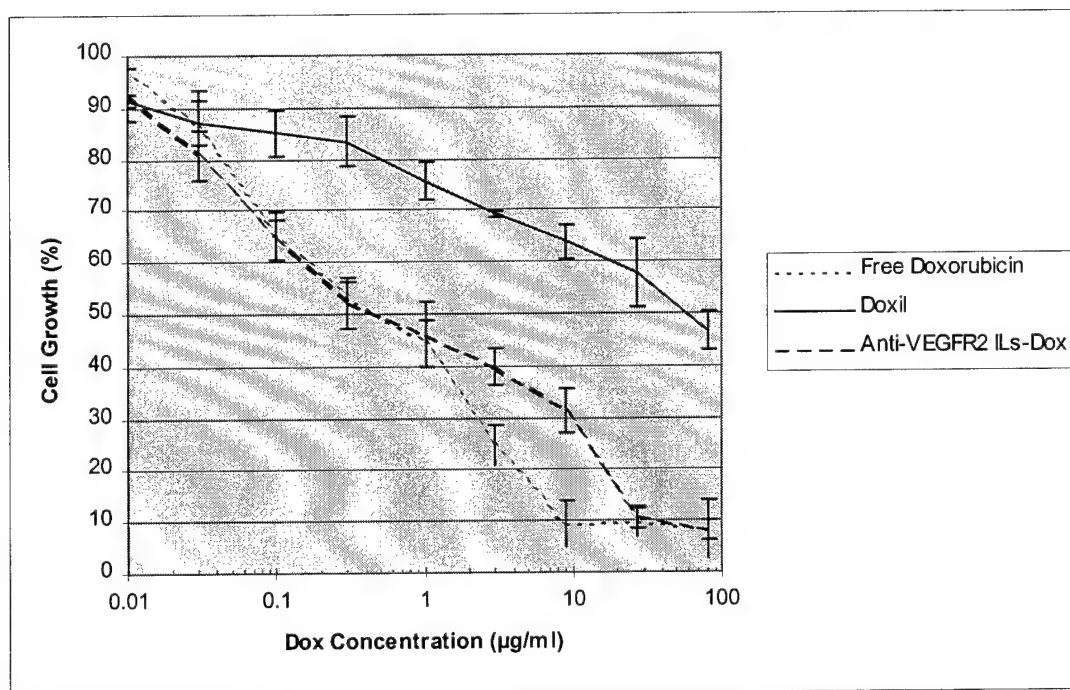
FIGURE 5B
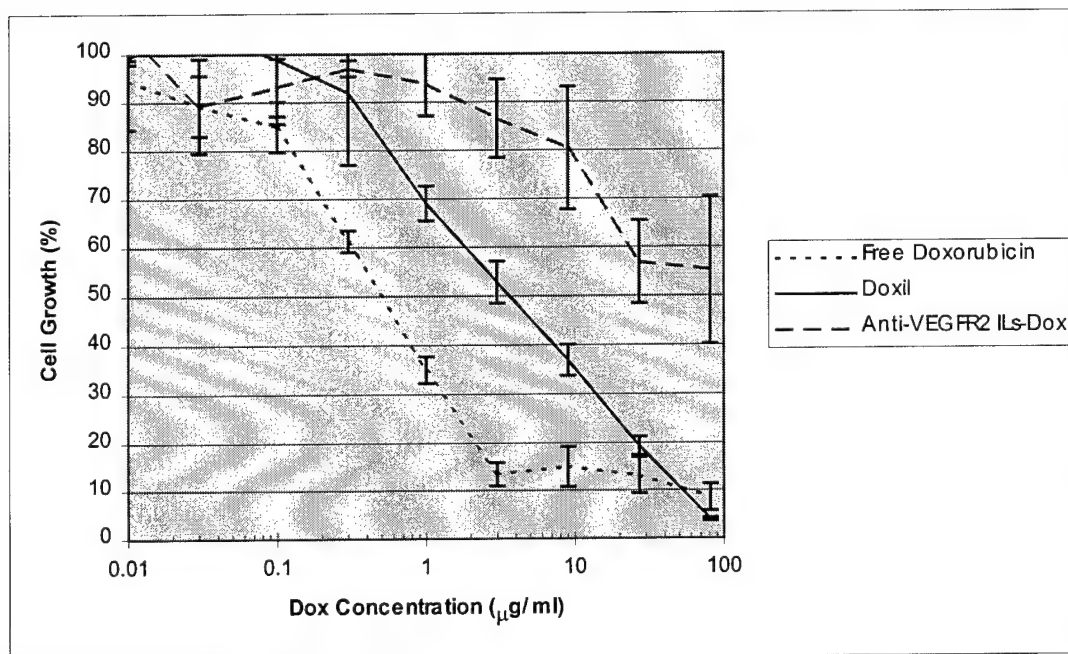
FIGURE 5C

FIGURE 5D

FIGURE 6A

FIGURE 6B